Bacillus vietnamensis sp. nov., a moderately halotolerant, aerobic, endospore-forming bacterium isolated from Vietnamese fish sauce

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Five strains of Gram-positive, endospore-forming, moderately halotolerant bacteria were studied taxonomically. Four were isolated from Vietnamese fish sauce and one from the Gulf of Mexico. Phylogenetic analysis based on 16S rRNA gene sequences showed that these strains clustered within the radiation of the genus *Bacillus* but separately from recognized *Bacillus* species. DNA G+C composition of the isolates ranged from 43 to 44 mol%. Strains 15-1^T and NRRL B-14850 showed high levels of DNA–DNA relatedness (82–100 %) to each other and to the other strains isolated here; they displayed low levels of DNA–DNA relatedness (< 29 %) to the type strains of selected recognized *Bacillus* species. They grew in 15 % NaCl and optimally in 1 % NaCl, which is characteristic of moderately halotolerant bacteria. The isolates grew at pH 6·5 to 10·0 but not at pH 6·0. Their cell walls contained *meso*-diaminopimelic acid. The major isoprenoid quinone was MK-7 and the principal cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{17:0}$. Based on these results, the strains tested were regarded as members of a novel *Bacillus* species for which the name *Bacillus vietnamensis* sp. nov. is proposed. The type strain is 15-1^T (=JCM 11124^T=NRIC 0531^T=NRRL 23890^T).

The ubiquity of *Bacillus* species and closely related bacteria in fermented fish products implied their importance in the preparation of these food materials. The results of several studies have supported this conjecture. For example, Itoh *et al.* (1993) verified the participation of bacteria in fish sauce fermentations. *Bacillus* strains have been isolated from fermented fish in Vietnam and Japan (Crisan & Sands, 1975). Researchers have isolated proteolytic *Halobacillus* (Choorit & Prasertsan, 1992) and *Bacillus* from fermented fish in Thailand (Chaiyanan *et al.*, 1999). Isolates of *Bacillus* species and moderately halophilic bacteria were recovered from Korean fermented seafoods (Sands & Crisan, 1974; Yoon *et al.*, 2001). *Bacillus* species have been isolated from nam-pla, a Thai fermented fish sauce (Saisithi *et al.*, 1966; Crisan & Sands, 1975). Mura *et al.* (2000) isolated four *Bacillus* strains from nuoc mam, a Vietnamese fish sauce; these isolates resembled *Bacillus firmus* phenotypically but showed a low level of DNA–DNA relatedness with the type strain of the species. Because these bacteria apparently originated mainly from marine fish, raw materials for production of fish sauce in Vietnam, the isolates were presumed to be halophilic or halotolerant. Yoon *et al.* (2003) have described two *Bacillus* species, *Bacillus marisflavi* and *Bacillus aquimaris*, isolated from sea water.

Preliminary comparisons of 16S rRNA gene sequences suggested a close relationship between the four above-mentioned *Bacillus* strains from nuoc mam and NRRL B-14850, an unidentified *Bacillus* isolate from the Gulf of Mexico (Siefert *et al.*, 2000). These five strains were studied to clarify their taxonomic position by 16S rRNA gene sequences, levels of DNA–DNA relatedness, DNA base compositions, chemotaxonomic properties and phenotypic characteristics. The data indicated that the five strains were members of a novel species, for which we propose the name *Bacillus vietnamensis* sp. nov.

Four strains (11-4, 15-1^T, 16-3 and 20-1) isolated by Mura *et al.* (2000) and strain NRRL B-14850 were used in this study. Reference strains used were *Bacillus amyloliquefaciens*

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Bacillus vietnamensis* strain 15-1^T is AB099708.

DSM 7^T, *Bacillus aquimaris* TF-12^T, *Bacillus atrophaeus* IFO 15539^T, *Bacillus firmus* JCM 2512^T, *Bacillus lentus* JCM 2511^T, *Bacillus licheniformis* JCM 2505^T, *Bacillus marisflavi* TF-11^T, *Bacillus mojavensis* NRRL B-14698^T, *Bacillus pumilus* JCM 2508^T, *Bacillus subtilis* JCM 1465^T and *Bacillus vallismortis* NRRL B-14890^T. Trypticase soy agar (Difco) was used as a basal medium and as a stock culture medium. Strains were stored at 4 °C.

Determination of the taxonomic relationships of the five isolates was based on 16S rRNA gene sequences. Sequencing was carried out using the methods reported by Shida *et al.* (1996) and Takagi *et al.* (1993). Sequences determined in this study were compared with 16S rRNA gene sequences obtained from EMBL/GenBank/DDBJ. Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc} value) (Kimura, 1980), construction of a neighbour-joining phylogenetic tree (Saitou & Nei, 1987) and a bootstrap analysis with 1000 replicates for evaluation of phylogenetic tree topology (Felsenstein, 1985) were carried out with CLUSTAL W version 1.6 (Thompson *et al.*, 1994). Alignment gaps and unidentified base positions were not taken into account for the calculations. The 16S rRNA gene sequence of strain 15-1^T (1200 bp) was determined. In addition, partial 16S rRNA gene sequences (300-base segments corresponding to positions 200–500) were determined for the three other *Bacillus* strains (11-4, 16-3, 20-1) isolated from nuoc mam. 16S rRNA gene sequence data of NRRL B-14850 (1544 bp) and other endospore-forming aerobic bacteria were obtained from DDBJ/EMBL/GenBank.

DNA was extracted and purified with the Qiagen Genomic-tip system. Levels of DNA–DNA relatedness were determined fluorometrically (Ezaki *et al.*, 1989). Probes were prepared with DNA from strain 15-1^T and NRRL B-14850. DNA base compositions were determined by HPLC (Tamaoka & Komagata, 1984).

Cellular fatty acid compositions, isoprenoid quinone compositions and isomers of diaminopimelic acid in the cell walls were determined by the methods of Komagata & Suzuki (1987).

Morphological, physiological and biochemical characteristics were determined by the methods described by Gordon *et al.* (1973) and Takagi *et al.* (1993). Growth in various salt concentrations was tested using trypticase soy broth (Difco) as the basal medium. Cells were cultivated in trypticase soy broth containing 1 % (w/v) NaCl with shaking. After cultivation for 24 h at 37 °C, 50 µl culture was added to 2·5 ml broth containing various NaCl concentrations and the inoculated broth was incubated on a reciprocal shaker (180 r.p.m.) at 37 °C. Growth was monitored spectrophotometrically at 660 nm. Other phenotypic characteristics were determined by using API 50 CH and 20 NE kits (bioMérieux) (Logan & Berkeley, 1984).

16S rRNA gene sequence similarity between strains 15-1^T and NRRL B-14850 was 98·6 %. The 300-base sequences of isolates 11-4, 16-3 and 20-1 were nearly identical to one another and to the corresponding segment of strain 15-1^T (data not shown). 16S rRNA gene sequence similarity values between strains 15-1^T and NRRL B-14850 and selected members of the genus *Bacillus* were less than 95·8 %. In a phylogenetic tree based on 16S rRNA gene sequences, strain 15-1^T and NRRL B-14850 formed a cluster with *B. aquimaris* and *B. marisflavi* within the genus *Bacillus* (Fig. 1); this cluster was linked to the *B. subtilis* group. 16S rRNA gene sequence similarities between strain 15-1^T and *B. aquimaris* TF-15^T and *B. marisflavi* TF-11^T were 99·4 and 98·6 %, respectively.

DNA–DNA relatedness values between strain 15-1^T and the four other isolates were 88–100 %; similarity values between NRRL B-14850 and the four isolates ranged from 82 to 100 %. Less than 29 % DNA–DNA relatedness was measured between strain 15-1^T or NRRL B-14850 and species belonging to the *B. subtilis* group, namely *B. amyloliquefaciens* DSM 7^T, *B. atrophaeus* IFO 15539^T, *B. firmus* JCM 2512^T, *B. lentus* JCM 2511^T, *B. licheniformis* JCM 2505^T, *B. mojavensis* NRRL B-14698^T, *B. pumilus* JCM 2508^T, *B. subtilis* JCM 1465^T and *B. vallismortis* NRRL B-14890^T, and marine *Bacillus* species *B. aquimaris* TF-12^T and *B. marisflavi* TF-11^T.

The isolates showed virtually the same cell morphology and phenotypic characteristics: cells were rod-shaped, measuring 0·5–1·0 by 2·0–3·0 µm, Gram-positive and aerobic. Ellipsoidal spores developed centrally in the cells and sporangia were not swollen. Cells were motile with peritrichous flagella. The strains tested produced catalase and oxidase.

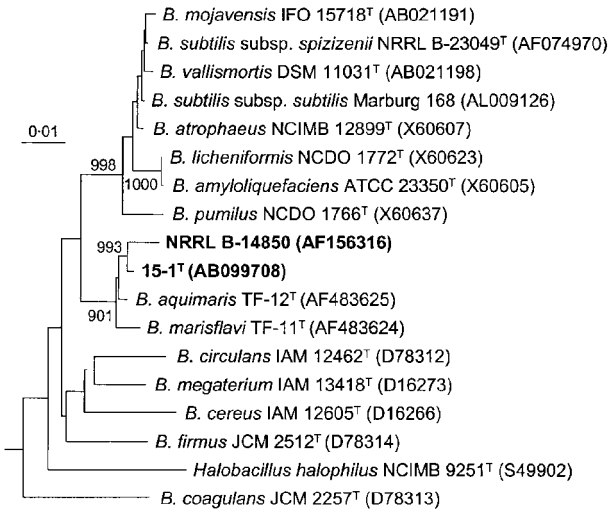

Fig. 1. Phylogenetic relationships of novel isolates and some aerobic endospore-forming bacteria on the basis of 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. The numbers indicate bootstrap values from 1000 resamplings. Bar, 0·01 nucleotide substitution per sequence position.

They grew in 15 % NaCl, and optimally in 1 % NaCl; they were therefore regarded as halotolerant bacteria. In addition, the isolates grew at pH 10·0 as alkaliphilic bacteria did but were phylogenetically distinct from these organisms (Fritze *et al.*, 1990; Li *et al.*, 2002). Other phenotypic characteristics are given in the species description below.

The isolates were differentiated from *B. firmus* based on the following characteristics: oxidase production, growth in 15 % NaCl and at 10 °C, resistance to lysozyme, hydrolysis of gelatin, aesculin, *p*-nitrophenyl *β*-D-glucopyranoside and DNA and assimilation of gluconate. The isolates were positive for these traits, whereas *B. firmus* was negative. Differential characteristics with *B. aquimaris* and *B. marisflavi* were growth at pH 4·5 and 9·0, hydrolysis of aesculin and starch and acid production from *N*-acetylglucosamine and inulin.

DNA G+C content of the five isolates ranged from 43 to 44 mol%. The major cellular fatty acids of the five strains tested were anteiso-$C_{15:0}$ (48·3±8·6 %), iso-$C_{15:0}$ (16·2±3·7 %), anteiso-$C_{17:0}$ (13·6±4·9 %) and iso-$C_{16:0}$ (11·2±1·8 %). Fatty acids occurring in minor amounts were iso-$C_{14:0}$ (3·7±1·8 %), $C_{14:0}$ (1·3±0·8 %), $C_{15:0}$ (1·0±0·5 %), $C_{16:0}$ (2·8±0·6 %) and iso-$C_{17:0}$ (1·3±1·0 %). The isolates contained menaquinone 7 (MK-7), which accounted for more than 82 % of the total menaquinones. *meso*-Diaminopimelic acid was found in the cell-wall peptidoglycan of the nuoc mam isolates. The above characteristics are traits frequently displayed by species of the genus *Bacillus* (Claus & Berkeley, 1986).

Phylogenetic studies showed that the nuoc mam isolates (11-4, 15-1^T, 16-3, 20-1) and NRRL B-14850 were members of a distinct group within the genus *Bacillus*. Low levels of DNA–DNA relatedness between the isolates and members of the closest neighbouring clade, consisting of *B. subtilis*-like organisms, supported the distinctiveness of the nuoc mam group. The nuoc mam isolates displayed characteristics typical of many *Bacillus* species, namely aerobic growth, spore production, major fatty acid composition of anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$, predominance of the MK-7 menaquinone, occurrence of *meso*-diaminopimelic acid in the cell wall peptidoglycan and DNA G+C content of 43–44 mol%. The isolates were regarded as moderately halotolerant bacteria because they could grow in the presence of up to 15 % NaCl and optimally at 1 % (Gilmour, 1990). This characteristic is generally regarded as typical of marine bacteria (Baumann & Baumann, 1981).

Based on these results, we consider that the five strains merit recognition as a novel species of the genus *Bacillus*, for which we propose the name *Bacillus vietnamensis* sp. nov.

Description of *Bacillus vietnamensis* sp. nov.

Bacillus vietnamensis (vi.et.nam.en′sis. N.L. adj. *vietnamensis* referring to Vietnam, the country where the type strain was isolated).

Cells are rod-shaped, measuring 0·5–1·0 by 2·0–3·0 μm, Gram-positive and aerobic. They are motile with peritrichous flagella. Ellipsoidal spores develop centrally in the cells and sporangia are not swollen. Catalase and oxidase are produced. Nitrate reduction, indole production, arginine dihydrolase and urease are negative. Growth occurs in the presence of lysozyme. Casein, starch, DNA, aesculin, gelatin, *p*-nitrophenyl *β*-D-galactopyranoside and tyrosine are hydrolysed. Production of hydrogen sulphide is not detected on trypticase soy agar. Acid is produced from glycerol, D-ribose, D-glucose, D-fructose, mannitol, *N*-acetyl D-glucosamine, aesculin, maltose, sucrose, trehalose, inulin, starch and glycogen; no acid is produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl α-D-xyloside, galactose, D-mannose (NRRL B-14850 produces acid from this sugar), L-sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl *β*-D-glucoside, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, melezitose, D-raffinose, xylitol, *β*-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, D-gluconate, 2-ketogluconate and 5-ketogluconate. Assimilation is positive for glucose, D-mannitol, *N*-acetyl D-glucosamine, maltose, gluconate and DL-malic acid, and negative for L-arabinose, D-mannose, n-capric acid, citrate and adipic acid. Growth occurs at 0–15 % (w/v) NaCl (optimum at 1 %). The isolates are regarded as moderately halotolerant bacteria. Growth occurs at 10–40 °C (optimum at 30–40 °C) (16-3 and NRRL B-14850 grow at 50 °C). Growth occurs at pH 6·5–10·0 but not at pH 6·0. DNA G+C content is 43–44 mol%. The major fatty acid is anteiso-$C_{15:0}$ (48·3±11·9 %), with lesser iso-$C_{15:0}$ (16·2±4·4 %). The major quinone is MK-7. *meso*-Diaminopimelic acid is found in the cell walls. Strains have been isolated from Vietnamese fish sauce and from the Gulf of Mexico.

The type strain is strain 15-1^T (=JCM 11124^T=NRIC 0531^T=NRRL 23890^T). The description of the type strain is the same as that of the species. DNA G+C content is 43 mol%. Major cellular fatty acids are anteiso-$C_{15:0}$ (51·4 %) and iso-$C_{15:0}$ (19·8 %). Isolation source is Vietnamese fish sauce.

References

Baumann, P. & Baumann, L. (1981). The marine gram-negative eubacteria: genera *Photobacterium*, *Beneckea*, *Alteromonas*, *Pseudomonas*, and *Alcaligenes*. In *The Prokaryotes*, vol. 1, pp. 1302–1331. Edited by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. G. Schlegel. New York: Springer.

Chaiyanan, S., Chaiyanan, S., Maugel, T., Hug, A., Robb, F. T. & Colwell, R. R. (1999). Polyphasic taxonomy of a novel *Halobacillus*, *Halobacillus thailandensis* sp. nov. isolated from fish sauce. *Syst Appl Microbiol* **22**, 360–365.

Choorit, W. & Prasertsan, P. (1992). Characterization of protease produced by newly isolated and identified proteolytic microorganisms from fermented fish (Budu). *World J Microbiol Biotechnol* **8**, 284–286.

Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. B. Holt. Baltimore: Williams & Wilkins.

Crisan, E. V. & Sands, A. (1975). Microflora of four fermented fish sauces. *Appl Microbiol* **29**, 106–108.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Felsenstein, J. (1985). Confidence limits on phylogenies; an approach using the bootstrap. *Evolution* **39**, 783–791.

Fritze, D., Flobdorf, J. & Claus, D. (1990). Taxonomy of alkaliphilic *Bacillus* strains. *Int J Syst Bacteriol* **40**, 92–97.

Gilmour, D. (1990). Halotolerant and halophilic microorganisms. In *Microbiology of Extreme Environments*, pp. 147–177. Edited by C. A. Edwards. Milton Keynes, UK: Open University Press.

Gordon, R. E., Haynes, W. C. & Pang, C. H.-N. (1973). *The Genus Bacillus*. Agriculture Handbook no. 427. Washington, DC: US Department of Agriculture.

Itoh, H., Tachi, H. & Kikuchi, S. (1993). Fish fermentation technology in Japan. In *Fish Fermentation Technology*, pp. 177–186. Edited by C. H. Lee, K. H. Steinkraus & P. J. A. Reilly. New York: United Nations University Press.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.

Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T. & Ezaki, T. (2002). *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. *Int J Syst Evol Microbiol* **52**, 1205–1209.

Logan, N. A. & Berkeley, R. C. W. (1984). Identification of *Bacillus* strains using the API system. *J Gen Microbiol* **130**, 1871–1882.

Mura, K., Maeda, H., Tanaka, H., Koizumi, Y. & Yanagida, F. (2000). Isolation of protease-productive bacteria from fish sauces collected in Vietnam. *Food Preserv Sci* **26**, 263–271 (in Japanese).

Saisithi, P., Kasemsarn, B. O., Liston, J. & Dollar, A. M. (1966). Microbiology and chemistry of fermented fish. *J Food Sci* **31**, 105–110.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sands, A. & Crisan, E. V. (1974). Microflora of fermented Korean seafoods. *J Food Sci* **39**, 1002–1005.

Shida, O., Takagi, H., Kadowaki, K. & Komagata, K. (1996). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* **46**, 939–946.

Siefert, J. L., Larios-Sanz, M., Nakamura, L. K., Slepecky, R. A., Paul, J. A., Moore, E. R., Fox, G. E. & Jurtshuk, P., Jr (2000). Phylogeny of marine *Bacillus* isolates from the Gulf of Mexico. *Curr Microbiol* **41**, 84–88.

Takagi, H., Shida, O., Kadowaki, K., Komagata, K. & Udaka, S. (1993). Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. *Int J Syst Bacteriol* **43**, 221–231.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Yoon, J.-H., Kang, S.-S., Lee, K.-C., Kho, Y. H., Choi, S. H., Kang, K. H. & Park, Y.-H. (2001). *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. *Int J Syst Evol Microbiol* **51**, 1087–1092.

Yoon, J.-H., Kim, I.-G., Kang, K. H., Oh, T.-K. & Park, Y.-H. (2003). *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **53**, 1297–1303.